Increased Sensitivity to Oxanosine, a Novel Nucleoside Antibiotic, of Rat Kidney Cells upon Expression of the Integrated Viral src Gene

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ABSTRACT

The mechanism of antitumor action of oxanosine was studied using a strain of rat kidney cells infected with a mutant Rous sarcoma virus, the src gene of which is temperature sensitive. Oxanosine inhibited cell growth in vitro, as well as nucleic acid synthesis in these cells, 10 times more strongly at a permissive temperature (33°C) than at a non-permissive temperature (39°C). Protein synthesis was inhibited only slightly at either temperature. The inhibition of cell growth and nucleic acid synthesis was reversed by guanosine, GMP, and to a lesser extent by adenosine and inosine. Oxanosine inhibited the conversion of [14C]hypoxanthine to guanine nucleotides in cells and again in the same temperature-related fashion. The conversion to adenine nucleotides was not inhibited. Oxanosine-5'-monophosphate was found to be a potent nearly competitive inhibitor, with respect to IMP, of IMP dehydrogenase (EC 1.2.1.14; IMP:NAD+ oxidoreductase) isolated from cells grown either at 33°C or at 39°C; with the former and the latter enzyme preparations, Ks's for IMP were 6.0 x 10^-6 M and 5.3 x 10^-6 M, respectively, while Ks for oxanosine-5'-monophosphate were 1-3 x 10^-6 M and 5.2 x 10^-6 M, as well.

INTRODUCTION

Oxanosine, a novel nucleoside antibiotic, has been reported to inhibit growth in vitro of some microorganisms and HeLa cells and to prolong the life span of the mice inoculated with L1210 leukemic cells (1). As a prescreening system for antitumor compounds, we have been using rat kidney cells infected with a mutant Rous sarcoma virus, the src gene of which is temperature sensitive; the cells are referred to as ts/NRK. Oxanosine, among various compounds tested, was found to inhibit the growth of ts/NRK more strongly at a permissive temperature (33°C) than at a non-permissive temperature (39°C) (2). We have observed that oxanosine and thymidine share a common membrane transport system which is more efficient in 33°C cells than in 39°C cells (2, 3). It is unlikely, however, that this is the sole reason for the selective growth inhibition of 33°C cells by oxanosine, because some compounds that are structurally unrelated to nucleosides show the same selective effects. Mycophenolic acid, an inhibitor of IMP dehydrogenase (4, 5), is among these compounds. Studies were initiated to find additional differences between 33°C cells and 39°C cells which could be responsible for the increased drug sensitivity of 33°C cells to oxanosine.

MATERIALS AND METHODS

Chemicals. Oxanosine and oxanosine-5'-monophosphate were obtained from Nippon Kayaku Co., Ltd., Tokyo, Japan. Decoyinine was obtained from Dr. Ochi, Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan. [8-14C]Hypoxanthine (54 μCi/mmol), [methyl-3H]thymidine (43 Ci/mmol), [5-3H]uridine (27.8 Ci/mmol), and L-[4,5-3H]leucine (55.5 Ci/mmol) were purchased from Amersham International, Buckinghamshire, England. Amine 336 (tricaprylyl tertiary amine), a water-immiscible amine, was obtained from Henkel Corporation, Kanakee, IL.

Cell Culture. The cells of a rat kidney line infected with ts25, a T-class mutant of Rous sarcoma virus Prague strain (ts/NRK) (6), were obtained from Dr. M. Yoshida, Cancer Institute, Tokyo, Japan. They were grown in Dulbecco's modified Eagle medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated calf serum (Grand Island Biological Co.) in 5% CO2 and humidified air. In this medium, ts/NRK cells grew with a generation time of 30-35 h until they reach a cell density of 1.3 x 10^6 cells/cm^2 at both temperatures (2). The cell number was counted in a Coulter counter after trypsinization. Dialyzed serum was used where indicated.

Colony Formation. For liquid cloning, cell layers were washed twice with Dulbecco's PBS, treated with trypsin, and squirted in the medium containing serum, and the resulting single-cell suspensions were diluted with the same medium to make 200 cells per ml. One-mL portions of the diluted cell suspension were added to culture dishes, each containing 2 ml of the prewarmed (33°C) medium. The dishes were then incubated at 33°C for 7-10 days without changing the medium. Colonies were washed free of the medium, fixed with 4% formalin in PBS, and stained with 0.1% of crystal violet. Colony-forming efficiency was about 40%.

For soft agar cloning, cells were suspended at 200 cells/ml of the medium containing 0.33% agar, 1-ml portions thereof were added to culture dishes, each containing a 4-ml agar layer consisting of the medium containing 0.5% agar. After the dishes were incubated for 2 weeks, the colonies consisting of more than 50 cells were scored. Colony-forming efficiency was about 30%.

Determination of the Rates of DNA, RNA, and Protein Syntheses. Ts/NRK cells from 33°C cultures were seeded at 2 x 10^4 cells/2 ml/35-mm dish and grown overnight at either temperature. Test compounds were added to the cultures 20 h after the cell seeding unless otherwise stated. Two h before cell harvest, 0.02 ml of [methyl-3H]thymidine (1 μCi/dish), [5-3H]uridine (1 μCi/dish), or L-[4,5-3H]leucine (1 μCi/dish) was added to each culture dish containing 1 ml of medium. In case of labeling with [3H]thymidine and [3H]uridine, cells were labeled for 1 h at each temperature, washed free of the radioactive medium with cold PBS, and received 2 ml/dish of cold 5% perchloric acid. The acid-insoluble residues of the cell layers were dissolved in 0.5 N KOH, mixed with a liquid scintillation solution, and submitted to radioactivity measurement. In the case of [3H]leucine, the labeled cell layers were scraped off with a rubber policeman and transferred into test tubes each containing 3 ml of 5% HCL.

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‡ The structure of oxanosine is 5-amino-3-β-D-ribofuranosyl-3H-imidazo[4,5-c]-[1,3]oxazin-7-one.

§ The abbreviation used is: PBS, phosphate-buffered saline.
perchloric acid, heated at 70°C for 15 min, and centrifuged, and the resultant precipitate was dissolved in 0.5 N KOH and counted. The rates of incorporation of \(^{3}H\)thymidine, \(^{3}H\)uridine, and \(^{3}H\)leucine into the acid-insoluble fraction of cells per h are denoted as the rates of DNA, RNA, and protein syntheses, respectively.

Incorporation of \(^{8}\)-Hypoxanthine into the Acid-Soluble Fraction of Growing ts/NRK Cells. Cell layers of ts/NRK in 35-mm dishes were prepared by cultivation at either permissive (33°C) or non-permissive (39°C) temperature for 1 day, washed 3 times with PBS containing 0.1% glucose (PBS-G), and incubated further at each temperature in 1.0 ml of PBS-G/dish. After 10 min, oxanosine of indicated amounts and \(^{3}H\)hypoxanthine (0.5 \(\mu\)Ci/ml, 20 \(\mu\)M final) were added, and the dishes were incubated for additional 15 min. The cell layers were washed with ice cold PBS-G and treated with 0.2 ml/dish of 2% perchloric acid at 0°C for 30 min. The acid extracts thereof were neutralized with equal amounts of formate buffers, pH 3.4, as follows: 0.5 M formate buffer to 2.5 cm above the sheets were developed with increasing concentrations of sodium formate, was applied as a 1-cm streak at 2.5 cm from the bottom of a sheet. A wick of Whatmann No. 3 MM paper was stapled to the top of carriers, was redissolved separately in 30 HIM potassium phosphate buffer (pH 7.4), dialyzed overnight against the same buffer, and stored at -20°C until use.

Enzyme Assays. IMP dehydrogenase was assayed by the spectrophotometric method (9). The reaction was performed in a quartz cell with a 1-cm path in a 1.0 ml reaction solution containing 150 \(\mu\)mol of Tris-HCl (pH 8.0), 3 \(\mu\)mol of reduced glutathione, 10 \(\mu\)mol of KCl, 4 \(\mu\)mol of NAD, 12 \(\mu\)mol of EDTA, 0.02 ml of enzyme solution, and disodium IMP at an indicated concentration. The reaction was started by rapid addition of prewarmed IMP and allowed to proceed at 37°C for 10–20 min. At the same time, the reaction was followed by measurement of the increase in 340-nm absorbance (NADH formation) against a blank which received no IMP. The 0 to 40% ammonium sulfate saturation fraction was used as an enzyme source. The initial velocity of the reaction was expressed as the change in 340-nm absorbance per min during the first 10 min of the reaction.

GMP synthetase assay was performed as follows (10). The reaction mixture contained, in a total volume of 0.25 ml, 60 \(\mu\)mol of Tris-HCl (pH 8.0), 12 \(\mu\)mol of MgCl, 1 \(\mu\)mol of ATP, 10 \(\mu\)mol of 1-glutamine, 0.02 ml of the 40 to 70% ammonium sulfate saturation fraction of the cell-free extract, and 1.5 \(\mu\)mol of xanthosine-5'-monophosphate. The reaction was started by the addition of enzyme solution to the rest of the reaction mixture. The control run proceeded without xanthosine-5'-monophosphate. The mixture was incubated at 37°C for 2 h. The reaction was stopped by the addition of 1.5 ml of 3% perchloric acid, and the precipitate was removed by centrifugation. The absorbance at 290 nm of the supernatant was determined in a quartz cell with a light path of 1 cm.

RESULTS

Growth Inhibitory Effect of Oxanosine on ts/NRK Cells and Reversal of the Inhibition by Purine Compounds. The growth-inhibitory activity of oxanosine against ts/NRK cells at permissive (33°C) and non-permissive (39°C) temperatures is shown in Chart 1. At 33°C, ts/NRK cells showed the transformed phenotype, while at 39°C, cells showed the normal-like one (data not shown). However, the cells grew at a comparable rate at the two temperatures (2). The oxanosine concentrations required for 50% growth inhibition from 3 separate experiments were 1–2 \(\mu\)g/ml and 10–30 \(\mu\)g/ml for 33°C cells and 39°C cells, respectively, indicating that oxanosine is 10 times more effective against the src-gene expressed cells (33°C cells) than against the src gene unexpressed cells (39°C cells). No such differences in toxicity between the two temperatures were observed with mouse leukemic L1210 cells, indicating that the differential effect was not simply due to possible instability of oxanosine at the higher temperature. The growth-inhibitory effect of oxanosine to 33°C cells was partially reversed by guanosine, GMP, and to a lesser extent by adenosine and inosine and not by xanthosine and the pyrimidine mixture, as shown in Table 1. Guanine was almost as effective as guanosine (data not shown). The lack of complete reversal by guanosine (and others, as well) should be ascribed to the toxicity of guanosine itself which is shown in Chart 2A. However, it should be noted that guanosine at 2 times the molar concentration of oxanosine almost abolished the cytotoxicity of oxanosine, as revealed by the colony formation assays (Chart 2, B and C).

Inhibitory Effect of Oxanosine on the Cellular DNA and RNA Syntheses and Reversal of the Inhibition by Guanosine. Oxanosine was added at a wide concentration range to growing ts/NRK cells at 33°C and 2 h later, 1 \(\mu\)Ci/ml of either \(^{3}H\)thymidine

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Reversal of growth inhibition of oxanosine by purine compounds

<table>
<thead>
<tr>
<th>Additions</th>
<th>Growth (% of control)</th>
<th>33°C*</th>
<th>39°C*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>14</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>Guanosine</td>
<td>54</td>
<td>63</td>
<td>65</td>
</tr>
<tr>
<td>GMP</td>
<td>62</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>Adenosine</td>
<td>48</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Inosine</td>
<td>39</td>
<td>39</td>
<td>39</td>
</tr>
<tr>
<td>Xanthosine</td>
<td>15</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Pyrimidine mixture</td>
<td>18</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

* Oxanosine concentration, 10 μg/ml.
* Oxanosine concentration, 100 μg/ml.
* Thymidine, uridine, and cytidine, 10 μg/ml each. Dialyzed serum was used.

Chart 2. Reversal of growth inhibition and cytotoxicity of oxanosine by guanosine. ts/NRK cells were treated with indicated concentrations of guanosine in the absence (O) or presence (•) of 10 μg/ml of oxanosine at 33°C. After 2 days of treatment, cells were trypsinized and prepared for the determination of (a) cell numbers, (b) liquid medium cloning, and (c) soft agar cloning as described in "Materials and Methods." Colony-forming efficiency was 40% for a and 30% for b, and these values were expressed as 100% in each graph. The values shown represent the means of duplicate dishes with a maximum range of 10%. Dialyzed serum was used.

or [3H]uridine, or [3H]leucine was added. After labeling for 1 h, the radioactivity incorporated into the acid-insoluble fraction of cells was determined. As shown in Chart 3, the syntheses of DNA and RNA were inhibited to a similar extent at various concentrations of oxanosine. The concentration required for 50% inhibition of the nucleic acid synthesis at 33°C was about 0.1 μg/ml. In contrast, the protein synthesis was not affected by oxanosine at concentrations up to 1 μg/ml. The inhibitory effect of oxanosine on the syntheses of DNA and RNA in 33°C cells was found to be 10 times stronger than the one in 39°C cells as shown in Chart 4. The correlation between the effect and the temperature was as what was observed with cell growth inhibition (Chart 1), indicating that the inhibition of the nucleic acid synthesis is the major cause of the cell growth inhibition by oxanosine. This notion was supported by the reversal effect of guanosine and some other purine compounds on the inhibition of nucleic acid synthesis by oxanosine (Table 2). Although adenosine and inosine appeared to be as effective as guanosine under these conditions, only guanosine was adequate if oxanosine concentrations were raised by 10 times (data not shown).

Inhibition of IMP Dehydrogenase by Oxanosine-5'-Monophosphate. The above results suggested that oxanosine might inhibit the de novo synthesis of guanine nucleotides. To confirm this, [14C]hypoxanthine was added to cultures simultaneously with or without oxanosine. After labeling for 15 min, the acid-soluble fraction of the cells was extracted and the purine nucleotides were separated by the chromatography using polyethyleneimine-cellulose, and their radioactivities were counted. As
Table 2
Reversal of nucleic acid synthesis inhibition of oxanosine by purine compounds
Concentrations of oxanosine: 1 µg/ml (33°C) and 10 µg/ml (39°C). Dialyzed serum was used.

<table>
<thead>
<tr>
<th>Nucleosides (10 µg/ml)</th>
<th>DNA (33°C)</th>
<th>DNA (39°C)</th>
<th>RNA (33°C)</th>
<th>RNA (39°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>20</td>
<td>56</td>
<td>34</td>
<td>44</td>
</tr>
<tr>
<td>Guanosine</td>
<td>94</td>
<td>88</td>
<td>93</td>
<td>96</td>
</tr>
<tr>
<td>GMP</td>
<td>65</td>
<td>94</td>
<td>58</td>
<td>87</td>
</tr>
<tr>
<td>Adenosine</td>
<td>97</td>
<td>88</td>
<td>94</td>
<td>88</td>
</tr>
<tr>
<td>Inosine</td>
<td>100</td>
<td>89</td>
<td>88</td>
<td>88</td>
</tr>
<tr>
<td>Xanthosine</td>
<td>20</td>
<td>60</td>
<td>33</td>
<td>42</td>
</tr>
<tr>
<td>Pyrimidine</td>
<td>12</td>
<td>49</td>
<td>28</td>
<td>35</td>
</tr>
</tbody>
</table>

Table 3
Inhibition of GMP synthetase from ts/NRK cells

<table>
<thead>
<tr>
<th>Compound (100 µg/ml)</th>
<th>ΔA_{360 nm/2 h}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>33°C</td>
</tr>
<tr>
<td>None</td>
<td>0.20 (100)*</td>
</tr>
<tr>
<td>Oxanosine</td>
<td>0.24 (120)</td>
</tr>
<tr>
<td>Oxanosine-5'-monophosphate</td>
<td>0.19 (95)</td>
</tr>
<tr>
<td>Decoyinine</td>
<td>0.02 (10)</td>
</tr>
</tbody>
</table>

*ΔA of the control run (including no test compound) at each temperature was taken as 100%. Numbers in parentheses indicate relative percent.

Chart 5. Effect of oxanosine on the synthesis of purine nucleotides from [14C]-hypoxanthine. ts/NRK cells were incubated at 33°C (○, △) or 39°C (▲, Δ) with varying concentrations of oxanosine and 20 µM [14C]-hypoxanthine (54 µCi/mmol). After 15 min, radioactivity in guanine nucleotides (○, △) and adenine nucleotides (▲, Δ) was determined as described in "Materials and Methods." The count of each nucleotide of the control run (without oxanosine) was taken as 100%. Each point represents the mean of separate analysis of duplicate dishes in one experiment, with a maximum range of 5%.

shown in Chart 5, the conversion of [14C]-hypoxanthine to guanine nucleotides, but not to adenine nucleotides, was found to be greatly reduced by the addition of oxanosine. Here again, the same correlation between the effects of oxanosine and the incubation temperatures was observed, indicating that the inhibition of guanine nucleotides is the major cause of the cell growth inhibition by oxanosine. Our next attempt was to find the target of oxanosine among individual enzymes involved in guanine nucleotide synthesis. Oxanosine was reported to inhibit GMP synthetase from Escherichia coli (11), so we tested if GMP synthetase from ts/NRK cells was also inhibited by oxanosine. However, as shown in Table 3, the mammalian enzyme showed no signs of inhibition by oxanosine, even at the concentration as high as 100 µg/ml (data not shown), oxanosine-5'-monophosphate inhibited the reaction significantly. Kinetic studies demonstrated that the inhibition of IMP dehydrogenase by oxanosine-5'-monophosphate was nearly competitive with respect to IMP, but precisely, the inhibition was of the mixed type (Chart 6). The K_m values calculated were 1–3 x 10^{-6} M and 5.2 x 10^{-6} M for the enzymes prepared from 33°C cells and 39°C cells, respectively, suggesting that the enzyme from 33°C cells were more sensitive than the enzyme from 39°C cells to oxanosine-5'-monophosphate. The enzymes showed similar K_m values, but the V_{max} of the enzyme from 39°C cells was about one-half of that from 33°C cells (Table 4).
TOXICITY OF OXANOSINE TO src-EXPRESSED CELLS

DISCUSSION

By determining various effects of oxanosine on ts/NRK cells at 33°C and 39°C, we found that the antibiotic inhibited guanine nucleotide synthesis, leading to cessation of cell growth. Oxanosine-5'-monophosphate inhibited IMP dehydrogenase. The mixed type inhibition of IMP dehydrogenase by oxanosine-5'-monophosphate (Chart 6) suggests that this nucleotide analogue binds not only to free enzyme as a false substrate, interfering with substrate binding, but also to the enzyme substrate complex, retarding the catalytic reaction. The inhibition of IMP dehydrogenase could explain the concomitant inhibition of DNA and RNA syntheses by oxanosine. The higher sensitivity of the enzyme from 33°C cells to oxanosine-5'-monophosphate than that from 39°C cells would be the basis, at least in part, of the selective toxicity of oxanosine to 33°C cells.

The same mechanism of action seems to apply to other tumor cells also, because IMP dehydrogenase activities in cell-free extracts from mouse leukemic L1210 cells and from Ehrlich ascites carcinoma cells were also inhibited by oxanosine-5'-monophosphate and not by oxanosine itself (data not shown). In E. coli cell extracts, oxanosine was reported to be an inhibitor of GMP synthetase (11). However, no inhibition of GMP synthetase isolated from ts/NRK cells or other tumor cells was observed, either by oxanosine or by its monophosphate. This discrepancy suggests the difference in sensitivity to oxanosine-5'-monophosphate between the enzymes from bacterial and mammalian origins.

The observed preferential cytotoxicity of oxanosine to tumor cells (33°C cells) than to normal cells (39°C cells) (Chart 1) was well correlated with the differential inhibitory effect on the nucleic acid synthesis and on the guanine nucleotide biosynthesis in these cells at the two temperatures. These results strongly suggest that the inhibition of GMP synthesis by interfering with IMP dehydrogenase is the major cause of the growth inhibition of tumor cells by oxanosine.

We have observed the correlation between the differential toxicity and the differential permeability of oxanosine against ts/NRK cells between the two temperatures (2). We would like to add another mechanism to the biochemical basis for the differential toxicity of oxanosine to tumor cells. The lower K₅₀ value of oxanosine-5'-monophosphate for the IMP dehydrogenase isolated from 33°C cells than that from 39°C cells also explains the higher sensitivity of tumor cells than normal cells to oxanosine. Moreover, other IMP dehydrogenase inhibitors, such as virazole (5, 14), bredisin (15), and mycophenolic acid (4, 5, 16) also exhibited the significant differential toxicity between the two temperatures (data not shown). Their differential effects were lesser than that of oxanosine, however. Other inhibitors of guanine nucleotide synthesis, such as 6-mercaptopurine, 6-thioguanine (17-19), 6-chloropurine riboside (16), and decoyinine (12, 13) did not show any differential toxicity.

We recently observed that herbimycin, an inhibitor of tyrosine protein kinase activity of p60src, caused rapid changes in cell morphology from "transformed" to "normal" (20). No such effect was observed with oxanosine, which suggests that the selective toxicity of oxanosine to 33°C cells does not include inhibition of src gene function itself. The alteration of IMP dehydrogenase and the thymidine transport system in such a way that the cells become more sensitive to oxanosine may be characteristic not only of src-induced tumor but of some other tumors because of its effectiveness in prolonging the life span of mice inoculated with L1210 (1), for instance.

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REFERENCES

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